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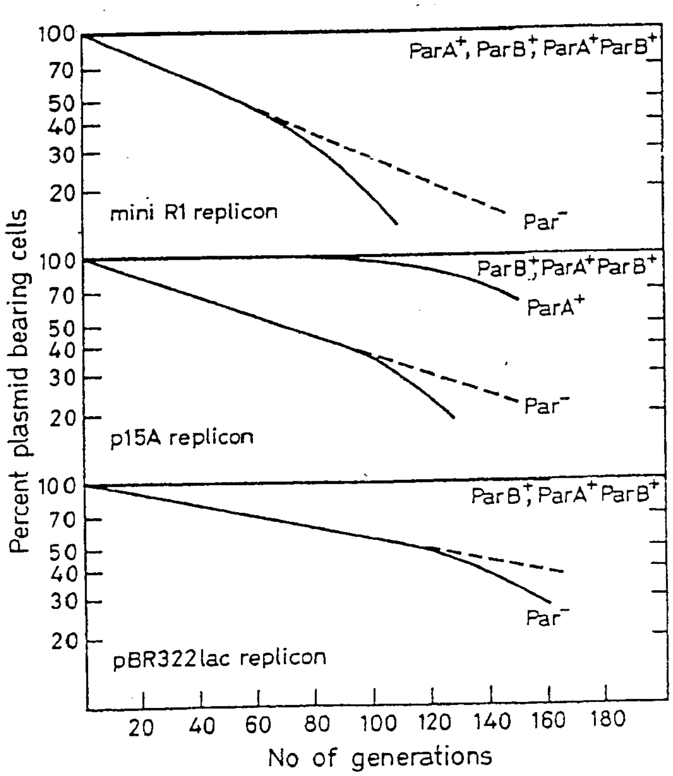
(57) Abstract

Plasmids which are in themselves unstably inherited or which have become unstable due to the insertion of a DNA fragment comprising one or more genes not naturally related to the plasmid are stabilized by means of a partitioning function exerted by a *par* region, especially a plasmid R1 *par* region, inserted into the plasmid on a DNA fragment which may be the length of the wild-type R1 *Eco*R1-A fragment, but which is preferably shorter than this fragment, and which may comprise the R1 *par* region A, the R1 *par* region B or both these R1 *par* regions. The stabilization obtained for several different types of plasmid, especially by employing both R1 *par* regions, approaches the stability level of wild-type plasmids, i.e. they typically have a frequency of loss of less than 5 x 10^{-6} per cell per generation. Such stabilized plasmids are useful in large-scale production of gene products as no particular bacterial strains or mutants are needed to secure plasmid maintenance, and as it is not necessary to employ a specific composition of the nutrient medium in which the host cells are grown in order to prevent loss of the plasmid from the bacterial population.



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STABILIZED PLASMIDS

The present invention relates to the stabilization of plasmids useful in the field of recombinant DNA technology for production of genes and their products.

TECHNICAL BACKGROUND

The continued maintenance of most natural plasmids, even in the absence of selection pressure (factors which secure the growth of only those organisms which harbour the plasmids; an example is an antibiotic in the nutrient medium in the case of a plasmid carrying a gene mediating resistance to the antibiotic), suggests that plasmid maintenance functions have been evolved to secure the continued presence of these extrachromosomal elements with high efficiency. The plasmid maintenance functions primarily consist of the replication genes including their control circuits which regulate the plasmid concentration in the cell. In growing cells the replication control system monitors the number of plasmid copies and corrects deviations from the average by increasing or decreasing the probability for plasmid replication. However, no replication control system can prevent the occurrence of cells with very few or just one copy of the plasmid, and from such cells the possibility of formation of a plasmid-free daughter cell is obvious. This problem is of course greatest for low copy number plasmids. Moreover, a passive distribution of plasmid molecules at cell division will inevitably result in a certain frequency of plasmid-free cells. Since the loss of a plasmid molecule from a cell is irreversible, the consequences of such an unstable situation will be that the entire population eventually becomes plasmid-free.

Besides a random distribution of plasmids at cell division, other factors may influence the rate of loss of plasmids from a culture of cells grown in the absence of selection for the maintenance of the plasmid. For example, some plasmids require a specific recombination system in order to resolve two newly replicated molecules (Austin et

al., *Cell 25*, 1981, pp. 729-36). In the absence of resolution, multimers will form (interlocked), and in this way even a high copy number plasmid may appear as a low copy number plasmid at the level of distribution to daughter cells, since the probability of generating plasmid-free cells will increase with increasing numbers of plasmid molecules interlocked in multimeric structures. Another phenomenon which is often observed in recombinant DNA technology is the conversion of a stable cloning vector into an unstable hybrid plasmid as a result of insertion of a DNA fragment, whose presence either causes a decrease in plasmid copy number or gives rise to a detrimental product that negatively interferes with cell growth.

In all such cases, plasmid segregation and loss occurs with a frequency (high or low) which cannot easily be controlled from the outside.

The stability of natural plasmids, and especially of low copy number plasmids, suggests that, in addition to the replication control system, a second set of maintenance functions exists which actively takes part in an ordered distribution of the plasmid molecules at cell division. Such functions have been termed partitioning functions, and studies, e.g., Meacock et al., *Cell 20*, 1980, pp. 529-42, Nordström et al., *Plasmid 4*, 1980, pp. 332-49, Seelke et al., *Plasmid 7*, 1982, pp. 163-79, have now shown that these are at least partly encoded by the plasmids themselves (in *par* regions). Thus, certain plasmid deletion mutants have lost their stable maintenance or inheritance despite their normal wild-type replication behaviour, indicating that a plasmid specified function securing stability has been removed. (cf. Nordström et al., *op.cit.*).

As many of the plasmids employed as vectors in recombinant DNA technology have been deleted of a large amount of DNA compared to the wild-type parent plasmid, they are susceptible to being unstably inherited. This poses a serious problem since instability of a plasmid will ultimately result in complete loss of the plasmid from the cells, and in any case will reduce the relative yield of plasmid-encoded gene products. This problem is particularly pronounced in large-scale

production of gene products where growth of microorganisms under selection pressure, e.g. an antibiotic, is normally not feasible and often at least undesirable from an environmental point of view, and where the microorganisms are grown for a large number of generations. Vectors which are only present in a few copies per cell are most likely to be unstably inherited and therefore lost from the cells. Even plasmids which ordinarily have a relatively high copy number securing a relative stability of the plasmid have been known to become unstable when DNA fragments carrying genes not naturally related to the plasmid are inserted therein.

DISCLOSURE OF THE INVENTION

The present invention relates to plasmids which carry an inserted gene or genes not naturally related to the plasmid and which additionally carry an inserted DNA fragment exerting a partitioning function. The term "inserted" is meant to indicate that the gene(s) or DNA fragment has been introduced into the plasmid at one stage during the construction of the final plasmid.

In the present context, the term "a partitioning function" denotes a function which ensures an ordered distribution of the plasmid molecules at cell division and which is encoded by a region of a plasmid which, dependent on the type of plasmid expressing the function, may comprise one or more genes. As mentioned above, such regions are found in naturally occuring, or wild-type, plasmids, but plasmids which express a Par^{+} phenotype, i.e. on which the partitioning genes are present, and which also carry an inserted gene or genes not naturally related to the plasmid are believed to be novel. In this context, plasmids are defined as naturally occurring extrachromosomal elements in microorganisms, which elements it is possible to isolate as such, or derivatives thereof.

The plasmids of the invention may be used as cloning or production vectors in the field of recombinant DNA technology for the purpose of obtaining a wide variety of products for technical or medical purposes

directly or indirectly mediated by the inserted gene or genes, particularly polypeptides and proteins or fragments thereof, enzymes, and a number of non-proteinaceous products of reactions of enzymes, low molecular weight products such as hormones, and nucleic acids; products of eukaryotic, especially mammalian, genes are of particular interest.

In accordance with a preferred embodiment of the present invention, the partitioning function is one which, in nature, is expressed by a region of the wild-type resistance plasmid R1, and therefore in the following referred to as an R1 *par* region. According to the present invention, such a region has been found to cause partial or full stability of not only unstable R1 miniplasmids carrying a gene or genes not naturally related to the plasmid, but also of frequently segregating plasmids not related to plasmid R1 carrying a gene or genes not naturally related to the plasmid. Another example of a particularly valuable partitioning function is that found on the wild-type plasmid F (Seelke et al., *op.cit.*) which also confers a high degree of stability to the recipient plasmid. In the following, reference will primarily be made to the R1 Par function, although it should be understood that many of the phenomena associated with the R1 Par function will also apply to other Par functions and that the general concept of the invention in its broad scope is not limited to the R1 Par function.

It has previously been indicated that the partitioning function of plasmid R1 is exerted by the so-called *Eco*R1-A fragment (cf. Nordström et al., *Plasmid 4*, 1980, pp. 332-49). In the course of research leading to the present invention, it has surprisingly been found that the *Eco*R1-A fragment which has a length of about 19 kb (19,000 base pairs) comprises two and only two distinct regions conferring a Par$^+$ phenotype on the recipient plasmid. These regions are situated at either end of the *Eco*R1-A fragment and no other DNA sequence in the *Eco*R1-A fragment is at present believed to have any plasmid stabilizing function. For the present purposes, the two regions have been designated the *par* region A and the the *par* region B, respectively, abbreviated as *par*A and *par*B, respectively.

As it is advantageous to operate with as small DNA fragments as possible, because small fragments are easier to insert into the plasmid and the resulting plasmids are easier to transform to host cells, one aspect of the present invention relates to plasmids which carry an inserted DNA fragment which is shorter than the *Eco*R1 fragment of plasmid R1 and contains an R1 *par* region. This inserted DNA fragment normally comprises, as its major component, the R1 *par* region A region, the R1 *par* region *B* region or both R1 *par* region A and R1 *par* region B. This means that substantially all of the DNA fragment inserted into the host plasmid is constituted by either or both of the *par* regions, and the remainder of the DNA on the fragments is present primarily to provide suitable restriction sites, providing the DNA fragments with the desired ends which are easily compatible with a corresponding or compatible restriction site on the recipient plasmid. Such ends may also be provided by means of linkers. It is, however, important to bear in mind that the DNA fragment comprising the *par* region A and the DNA fragment comprising the *par* region B may be introduced separately and sequentially into the same plasmid which will then be phenotypically identical with a plasmid in which the phenotype $ParA^+$, $ParB^+$ has been established by inserting the *par* region A and *par* region B on the same DNA fragment. For instance, if it is desired to further stabilize a plasmid already carrying a *par* region, such as the *parB* region, the plasmid may be restricted with an appropriate restriction enzyme and a DNA fragment having ends which are compatible with this restriction site and carrying the other *par* region, such as the *parA* region, may then be inserted. The opposite procedure, i.e. insertion, in a similar manner, of a *parB* region on a plasmid already carrying a *parA* region, may also be employed.

In accordance with this, interesting plasmids are plasmids in which the inserted DNA fragment comprising R1 *par* region A and R1 *par* region B has a length not exceeding about 6 kb, especially not exceeding about .4 kb, in particular not exceeding 3 kb. When the inserted DNA fragment includes the R1 *par* region A, it will normally have a length not exceeding about 4 kb, especially not exceeding about 2.5 kb, in particular not exceeding about 2 kb. When the

inserted DNA fragment comprises the R1 *par* region B, it will normally have a length not exceeding about 2 kb, especially not exceeding about 1.5 kb, in particular not exceeding about 1 kb. The preference for small DNA fragments for the reasons stated above should not, however, exclude the possibility of obtaining plasmid stability by inserting the entire *Eco*R1-A fragment; this may for instance be acceptable where the size of the plasmid to be stabilized is less critical. If the fragment is inserted *in toto*, it may be advantageous for screening purposes to provide the DNA fragment with a gene mediating antibiotic resistance. If, however, for some reason, it is desired to reduce the size of the *Eco*R1-A fragment, this may be done in various ways, such as by partial restriction of the *Eco*R1-A fragment with the restriction enzyme *Pst*I, or by excising each R1 *par* region from the large fragment, and transferring each region sequentially to the same DNA fragment which may then be inserted into the plasmid to be stabilized.

The plasmids which are stabilized in accordance with the principles of the invention may be either plasmids which have a natural relation to the partitioning region inserted, such as R1 miniplasmids which are stabilized by means of an inserted R1 *par* region (R1 miniplasmids have been deleted of much of the original R1 DNA and do consequently not usually in themselves contain an R1 *par* region), or they may be plasmids which do not have a natural relation to the partitioning function. It is interesting to note that efficient partitioning functions useful according to the present invention are capable of securing a satisfactory stability not only of plasmids to which they are naturally related, such as in the case of the stabilization of R1 miniplasmids with an R1 *par* region, but also of plasmids to which the partitioning function is not naturally related. An example of the latter is the insertion of an R1 *par* region in a non-R1 plasmid, such as a pMB1 plasmid or a derivative thereof, such as a pBR322 plasmid or a derivative thereof (these plasmids are ordinarily stable, but are susceptible to becoming unstable when a gene or genes not naturally related to the plasmid are inserted). Another example is the use of an R1 *par* region for stabilizing certain plasmid types which, at least at one stage during the cultivation of bacteria harbouring the plasmid,

have a low copy number, e.g. a copy number of 0.5-5 copies per cell, such as low copy number broad host range plasmids (plasmids which are able to replicate in many different host strains or species; this class includes the so-called shuttle vectors which are able to replicate in two or more types of microorganism) and derivatives thereof, e.g. RK2. Apart from R1, other plasmids of the incompatibility group IncFII requiring stabilization include, e.g., R100 and R6. The use of an R1 *par* region for the stabilization of unstable plasmid F derivatives also lies within the scope of the present invention.

One type of plasmids for which a stabilization in accordance with the present invention is important is conditional runaway replication plasmids, i.e. plasmids which, when host microorganisms harbouring the plasmids are grown under certain conditions, have a constant low plasmid copy number, and which, when the microorganisms harbouring the plasmid are grown under certain different conditions, lose their replication control so that the plasmid copy number increases exponentially until the host cell ceases to grow. Runaway replication plasmids for which the stabilization according to the invention is particularly vital are runaway replication plasmids with a copy number not exceeding about 3-5 copies per cell, and especially such plasmids, the copy number of which does not exceed about 0.5-1 copy per cell (the figure 0.5 is understood to mean that the frequency of replication is less than one per cell cycle) when the microorganisms harbouring the plasmids are grown under those conditions which ensure such a low plasmid copy number and which, when the host microorganisms are grown under certain different conditions ensuring a substantially increased plasmid copy number, has a copy number in the range of at least about 500-1000 copies per cell.

The low plasmid copy number under certain conditions (typically a low temperature such as a temperature of about 30°C) is desirable when plasmids are used as vectors for the insertion of foreign genes coding for products which are partially toxic or lethal to the host cell, as due to the low replication rate of the plasmid at, e.g., a low temperature, the genes are only expressed in small amounts, if at all, so that the cells remain undamaged in the propagation stage of the

culture. However, in plasmids with such an extremely low copy number as that stated above under propagation stage conditions, such as growing cells at a low temperature, the lack of a *par* region results in loss of a plasmid from the microbial population with a frequency of about 1% per generation for plasmids with a copy number not exceeding 3-5 copies per cell when these are grown under the conditions securing such a low copy number. The frequency of loss for plasmids with a copy number not exceeding about 0.5-1 copy per cell is about 5%/cell/generation. It is obvious that without any partitioning function to stabilize them, the plasmids would be lost from the cells before the cells had reached such a density in the nutrient medium that it would be economic to induce runaway replication; this is particularly the case in large-scale production requiring many hundreds of generations of cell growth to reach production size culture.

Such runaway replication may be made conditional by inserting a regulatable promoter upstream of the native replication control gene(s) of the plasmid (a detailed description of this phenomenon is found in Applicant's co-pending application entitled "Plasmids with Conditional Uncontrolled Replication Behaviour" and filed on the same day as the present application). Plasmids which have a runaway replication behaviour may be of many different types, but preferred runaway replication plasmids are R1-type plasmids.

In the present specification, the term "stability" (and related terms) is intended to mean a frequency of loss of the plasmid from the host cell of less than 2×10^{-4} per cell per generation. In fact, it is possible to obtain plasmids which are as stable as wild-type plasmids, i.e. with a frequency of loss of less than 3×10^{-6} per cell per generation, which corresponds to the level of mutation rate of genes, by incorporating a Par function in the plasmid. This latter frequency of loss (LF) value is evident when plasmids are stabilized by both R1 *par* region A and R1 *par* region B, such as when the entire *Eco*R1-A fragment has been inserted in the plasmids.

As mentioned above, the Par^{+} phenotype of plasmid R1, however, has been localized to each separate *par* region on the *Eco*R1-A fragment.

It has been found that each of these *par* regions exert a stabilizing effect on unstable plasmids, which may be defined as a plasmids lacking a stabilizing, or partitioning, function. Such plasmids which phenotypically are Par^- are usually lost from the host cell with higher or lower frequency; thus, for instance plasmid R1 derivatives deleted of the *par* region are lost from the host cell with a frequency of about 1.5×10^{-2} per cell per generation. Similarly, plasmid p15 derivatives, which are Par^- are lost with a frequency of about 1×10^{-2}/cell/generation, and some plasmid pMB1 (pBR322) derivatives (such as the one described in Example 3:3) may be lost with a frequency of about 6×10^{-3}/cell/generation. Conversely, R1 plasmids stabilized with either *par*A or *par*B alone have an LF value of about 10^{-4} per cell per generation, corresponding to a 100-fold stabilization when a DNA fragment carrying either of these *par* regions is inserted into an unstable vector; the figures for a corresponding p15 derivative are about 8×10^{-4} (Par A+), 1×10^{-6} (Par B+), and the figures for a corresponding pMB1 derivative are 5×10^{-5} (Par B+). Plasmids which carry both the *par A* and the *par B* region have an LF value of approximately 10^{-6} per cell per generation, or a 10^4-fold stabilization. For easy reference, the results for stabilized and unstable replicons of different origins are shown in table 1. This indicates that each *par* region acts independently of the other, that the *par* regions are approximately equally efficient in stabilizing at least R1 and p15 plasmids and that their action or effect is cumulative. This finding may be utilized when determining the degree to which an unstable plasmid need be stabilized. If a less drastic stabilization is required, i.e. if it is estimated that the plasmid to be stabilized is not excessively unstable (with an LF value of less than 10^{-2}/cell/generation), it may be sufficient to insert a DNA fragment containing one of the *par* regions in order to obtain a satisfactory stability, i.e. prevent a gradual loss of the plasmid from a large-scale production population of bacteria over several hundred generations. On the other hand, if the plasmid is very unstable, (with an LF value of more than 10^{-2}) it may be necessary or at least advantageous to insert both *par* regions in order to secure extreme stability of the plasmids.

Table 1

Frequencies of loss/cell/generation of different replicons

Type of Replicon	Frequencies of loss x 10^{-4}/cell/generation			
	Par Phenotype			
	Par^-	$ParA^+$	$ParB^+$	$ParA^+$ $ParB^+$
R1	150	0.6	1.0	0.04
p15	100	8.0	0.01	0.01
pMB1 (pBR322)	60	ND[1]	0.5	0.1

[1] ND = no data

These measurable LF values may be utilized when plasmids of any of the types defined above are to be used as vectors in the production of gene products, and should thus contain at least one gene not naturally related to the plasmid. A further aspect of the present invention thus relates to a method of producing a gene product from plasmid DNA in which bacteria harbouring a *par*-stabilized plasmid with any of the above-described characteristics are cultivated and the gene product of the plasmid is harvested from the bacterial culture. The cultivation *per se* is suitably performed using conventional techniques including conventional nutrient media which are known to be optimal to the bacterial species in question. It is worth noting that due to the stabilization, a specific composition of the nutrient medium is not needed. Also, the harvesting of the gene product is performed in accordance with well-known methods adapted to the identity and properties of particular gene product prepared, the properties of the host bacterium, etc. The cultivation is continued for at least 100 generations of the bacteria; in large-scale production, the number of cell generations needed to propagate the bacteria may exceed 100 generations. Under these circumstances, the LF value of the plasmid will be so selected by the presence of a *par* region therein that the loss of the plasmid is less than 2 x 10^{-4}/cell/ generation. This LF

value will usually be attainable by means of one *par* region alone. In some cases it is, however, preferred to obtain LF values for the plasmid of less than 10^{-5}/cell/ generation, in particular less than 5 x 10^{-6}/cell/generation.

Although these very low LF values may in some instances be attainable by the insertion of only one *par* region (particularly R1 *par* region B), they will usually need the presence of both R1 *par* regions.

A further aspect of the invention relates to bacteria harbouring plasmids of the above-defined type. It is a particular advantage of the plasmids of the invention that no particular mutants or strains are needed to secure plasmid maintenance. Thus, any species and strain of bacterium capable of harbouring such plasmid may be employed, such as gram-negative bacteria. A specific example of a bacterium in which plasmids according to the present invention are able to replicate and maintain their stability is *Escherichia coli*.

Finally, the present invention relates to a DNA fragment which comprises, as its major component, an R1 *par* region. This implies that substantially all of the DNA fragment inserted into the host plasmid is constituted by either of both of the par regions, and the remainder of the DNA is present to provide suitable restriction sites for insertion with a compatible restriction site on the recipient plasmid. The inserted DNA fragment comprising R1 *par* region A and R1 *par* region B should, in accordance with this principle, have a length not exceeding about 6 kb, especially not exceeding about 4 kb, in particular not exceeding 3 kb. When the DNA fragment includes the R1 *par* region A, it will normally have a length not exceeding about 4 kb, especially not exceeding about 2.5 kb, in particular not exceeding about 2 kb. When the DNA fragment comprises the R1 *par* region B it will normally have a length not exceeding about 2 kb, especially not exceeding about 1.5 kb, in particular not exceeding about 1 kb. It has surprisingly been found that such small, and therefore easily insertable, DNA fragments have retained their stabilizing function, as, by restriction enzyme mapping, the *par*A region has been nar-

rowed down to a region with a length of about 1800 bp (base pairs), and the *par*B region has been narrowed down to about 900 bp. It is possible that the gene or genes actually providing a Par$^+$ phenotype are even smaller.

A serious problem in the construction hybrid plasmids with a Par$^+$ phenotype has been the lack of a fast and simple method of screening for this phenotype. In general, the proper hybrid plasmids may be identified by the Par$^+$ phenotype which results in high stability of the plasmid during growth of the plasmid without selection pressure. However, this type of screening is tedious, and in any case it is only relevant if the parent plasmid is unstably inherited. Alternative screening methods have now been developed as outlined below.

a) *Screening for insertion of parA+ fragments*

If two different plasmids (from separate incompatibility groups) both carrying the *parA*$^+$ region are present in the same cell they will dislodge each other with a certain frequency (incompatibility), most likely because they compete for the partition apparatus in the cell. This type of *par* mediated incompatibility phenotype can be exploited in screening for *par* hybrids. For instance, a ParA$^+$ plasmid may be transformed to a Δ*lac E. coli* strain (e.g. CSH50) harbouring another plasmid carrying the *lac* genes and the *parA*$^+$ region. Normally the incoming plasmid will exert *par*$^+$ mediated incompatibility against the resident ParA$^+$ plasmid. Due to the Lac$^+$ phenotype of the resident plasmid such an incompatibility is easily detected if transformants are selected on the basis of a resistance marker found only on the incoming plasmid, whereas the presence or absence of the resident plasmid is scored on indicator substrates such as McConkey lactose plates. Replica plating colonies from such plates to new similar plates will reveal even low levels of dislodgement of the resident plasmid as colourless colonies showing the occurrence of Lac$^-$ cells. A further stability test or a more extensive incompatibility test may be required to test the properties of potential *parA*$^+$ hybrid plasmids. In this way it is possible - by involving the proper (compatible) combination of incoming and resident plasmids - to quickly screen for insertion of Inc$^+$ (Par$^+$) fragments in any plasmid whether it is unstable or stable.

b) *Screening for insertion of parB+ fragments*

Since it has also been demonstrated that two unrelated plasmids which both carry the *parB*$^+$ region are incompatible with each other the same screening strategy as described for the construction of *parA*$^+$ hybrids applies to the constructions of *parB*$^+$ hybrids.

c) *Screening for insertion of parA+ ,B+ fragments*

Besides having the potential of dislodging both *parA*$^+$ and *parB*$^+$ hybrid plasmids according to the descriptions above, the *Eco*R1-A fragment with the Tn5 insertion allows a direct selection (Km^R) for plasmids carrying the *parA*$^+$,*parB*$^+$ fragment. Thus, despite the large size of the fragment even very few hybrids are easily selected for. A smaller DNA fragment comprising both *par*$^+$ regions will of course also exert incompatibility against both *parA*$^+$ and *parB*$^+$ plasmids.

DESCRIPTION OF THE DRAWING

Reference is made to the drawing in which
Figs. 1-5 and 7-17 show restriction maps of the plasmids described in the examples,
Fig. 6 shows a detailed map (not drawn to scale) of the *Pst*I-D fragment from R1, and
Fig. 18 shows stability curves of different types of replicon carrying different *par* regions compared to Par^- replicons.

In Figs. 1-5 and 7-17, linear restriction maps of the plasmids described in the examples are shown in which the phenotypes and genotypes of the plasmids are indicated above the horizontal line which denotes the parent plasmid. Thus, *parA* represents one of the regions of plasmid R1 securing plasmid maintenance, *parB* represents the other region of plasmid R1 securing plasmid maintenance; *ice*I represents a gene mediating immunity to colicin E1; *ori* or *oriV* represents the origin of plasmid replication; *bla* represents a gene encoding resistance to ampicillin; IR represents an inverted repeat structure on Tn5; Km^R represents kanamycin resistance; Cm^R represents chloramphenicol resistance; Ap^R represents ampicillin resistance; Tc^R represents tetracyclin resistance; *repA* represents a gene coding for a protein required for R1 replication; *lacZ*, *lacY* and *lacA* represent the inserted *lac* operon of which *lacZ* codes for β-galactosidase, *lacY* codes for permease and *lacA* codes for transacetylase; *repA-lacZ'* represents a fusion between the *repA* and *lacZ* genes; *cop*B represents a gene coding for a polypeptide which represses transcription from the *repA* promoter (of R1 plasmids); *copA* represents a gene coding for an RNA molecule that inhibits translation of RepA-RNA; cI_{857} represents a gene which codes for a temperature-sensitive λ repressor controlling λP_R promoter activity; Pdeo represents the *deo* promoter. The arrow denotes the direction of transcription and the "triangles" denote insertions of DNA. The filled-in areas and the blank areas denote inserted genes; the dotted line denotes a deletion.

Below the horizontal line, the sites for restriction enzymes are shown in which E denotes *Eco*R1; P denotes *Pst*I; B_1 denotes *Bam*HI except in Figs. 1 and 5 where, outside the Tn5 DNA, it denotes *Bal*I; H_1 denotes *Hpa*I; E_V denotes *Eco*RV; B_2 denotes *Bgl*II; S_1 denotes *Sal*I; H_3 denotes *Hind*III; and C denotes *Cla*I.

In Fig. 6, the *Pst*I-D fragment has been mapped further; the numerals above the horizontal line indicate the number of base pairs between the restriction sites. Below the horizontal line, the sites for restriction enzymes are shown in which R_1 denotes *Rsa*I; P denotes *Pst*I; and H_1 denotes *Hpa*I.

In Fig. 18, stability curves are shown for the R1, p15 and pBR322 derivatives constructed and tested in the examples. Each curve represents the average of at least two liquid cultures followed for more than 100 generations. It appears from the figure that plasmids containing both *parA* and *parB* are extremely stably inherited, which is also the case with plasmids containing *parB* alone and with mini-R1 plasmids stabilized with *parA* alone.

It appears from the curves for Par^- plasmids that initially the frequency of plasmid-carrying cells decreases exponentially, as expected, because of a constant rate of loss. At later stages the frequency of plasmid-carrying cells appears to decrease faster (indicated with the full lines) due to a slightly faster growth rate of plasmid-free cells. The dotted line indicates the curve adjusted with respect to this faster growth rate to show the actual frequency of loss.

In contrast, plasmids which are phenotypically Par^- are lost with a frequency of 1.5×10^{-2}/cell/generation for R1 plasmids, 1×10^{-2}/cell/ generation for p15 plasmids and 6×10^{-3}/cell/generation for some pBR322 plasmids, an example of which is described in Example 3.3. In fact, the loss frequencies of the p15 Par^- and pBR322 Par^- derivatives are surprisingly high when the copy numbers of these vectors are considered. For example, the copy number of p15 is of the order of 15-20 per cell, predicting a loss rate of 10^{-9}/cell/ generation if binomial distribution of the plasmids is assumed. Nevertheless, the high loss rate observed is readily explained by the fact

that p15 replicons form *recA* dependent cointegrates, presumably because of the lack of a *loxP*-like resolution function (Austin et al., *Cell 25*, 1981, pp. 729-36). Also pBR322 derivatives are known to form cointegrates and when the copy number decreases because of e.g. large cloned fragments, a considerable instability results.

MATERIALS AND METHODS

The strain of *Escherichia coli* K-12 used was CSH50 (Δ *pro-lac, rpsL*; cf. J. Miller: *Experiments in Molecular Genetics*, Cold Spring Harbor, New York, 1972). Several plasmids and bacteriophages were used (Table 2).

The experimental techniques used were standard techniques employed in the fields of microbial genetics (J. Miller: *Experiments in Molecular Genetics*, Cold Spring Harbor, New York, 1972) and genetic manipulation (Davis, Botstein and Roth: *A Manual for Genetic Engineering*; *Advanced Bacterial Genetics*, Cold Spring Harbor, New York, 1980).

All cells were grown in LB medium (Bertani, *J. Bact 62*, 1951, p. 293) with 0.2% of glucose and 1 µg/ml of thiamin, or A+B minimal medium (Clark and Måløe, *J. Mol. Biol. 23*, 1967, p. 99) supplemented with 0.2% of glucose and 1% casamino acids. The plates used were LA plates containing LB medium and 1.5% of agar.

McConkey lactose indicator plates were prepared as recommended by the manufacturer (Difco), and X-gal plates were prepared by adding 20-40 µg/ml of 5-bromo-4-chloro-indolyl-β-D-galactoside to A+B minimal medium supplemented with 0.2% of glucose and 1 µg/ml of thiamin.

Physico-chemical methods

Clear lysates were prepared according to the method described by Clewell and Helinski, *Proc. Natl. Acad. Sci. USA 62*, 1969, pp. 1159-66.

Small scale preparation of plasmid DNA was performed by the method of Birnboim et al., *Nucl. Acids Res. 7*, 1979, pp. 1513-23.

Large scale preparation and analysis of plasmid DNA was performed using dye boyant density gradient centrifugation according to Stougaard and Molin, *Anal. Biochem. 118*, 1981, p. 181.

Polyacrylamide gel electrophoresis and agarose gel electrophoresis of DNA preparations was carried out essentially as described by Molin and Nordström, *Methods in Plasmid Biology*, Odense University 1982.

The restriction endonucleases were used in accordance with the prescriptions provided by the manufacturer (Boehringer, Mannheim or Biolabs, New England) at 37°C. Double and triple digests were performed by starting with the enzyme requiring the lowest salt concentration and then adjusting with additional buffer before adding the next enzyme.

Treatment with the exonuclease *Bal*31 was performed as follows: 0.1 unit of *Bal*31 was added to 50 µg linear DNA and samples were taken out at 1', 2', 4', 8', 16', 32' and 60' to 60 mM EDTA, extracted with phenol, ethanol precipitated and resuspended in 20 µl TE buffer. Half of the 20 µl was digested with the appropiate restriction enzyme subjected to agarose gel electrophoresis to determine the average size of the deleted DNA deletions. To the other half, the appropriate linker was added and the mixture ligated for 48 hours with an excess of T4 DNA ligase.

Ligation of restricted plasmid DNA was performed as recommended by the manufacturer with the exception of blunt end ligation, where an excess of T4 DNA ligase and ATP was added.

Microbiological methods

Partitioning test I: The construction of Lac^{+} vectors made it possible to determine the Par^{+} phenotype of a plasmid simply by streaking on nonselective McConkey lactose plates or X-Gal plates. Bacteria (Δ *lac*)

harbouring these plasmids mediate a Lac^+ phenotype easily scored as coloured colonies on the indicator plates, while plasmid-free cells have a Lac^- phenotype and appear as colourless colonies.

Partitioning test II: (Used for Lac^- plasmids): A colony from a selective plate (a plate containing an antibiotic) was streaked on another selective plate. From this plate, one colony was streaked on an LA plate so as to form single colonies. From the LA plate approximately 10 colonies were suspended in 1 ml of 0.9% of NaCl to a dilution of 10^{-4} and 10^{-5}, respectively. 0.1 ml of the 10^{-4} and 10^{-5} dilutions were spread on LA plates. From these plates, the resistance pattern of 50 colonies (200 colonies if a weak instability is expected) were tested on the appropiate selective plates. The frequency of loss (LF value) is then calculated on the basis of the formula

$$LF = 1 - (\nu)^{(1/27)}$$

where ν is the frequency of plasmid-bearing cells and assuming that one colony grows for 27 generations. Inherent in this method is a large statistical fluctuation.

Partitioning test III: Quantitative measurements of the stability of Lac^+ and Lac^- plasmids. One complete colony was taken from a selective plate and resuspended in 1 ml of 0.9% of NaCl to a concentration of 10^8 cells/ml. 2x0.1 ml of the 10^{-3} dilution were used to inoculate 2x10 ml of LB medium and inoculation was performed at 30°C with shaking. At a cell density of about 5 x 10^8 cells/ml, the cultures were diluted 10^4 and 10^5 fold. 0.1 ml of the 10^4 dilution (5 x 10^3 cells) was used to inoculate 10 ml of fresh LB medium and 0.4 ml of the 10^5 dilution was spread on McConkey lactose plates, and the plates were inoculated at 30°C overnight. The dilution from 5 x 10^8/ml to 5 x 10^2/ml corresponds to 20 generations of growth (2^{20}), so the change in the frequency of plasmid bearing cells from one dilution to the next corresponds to that occurring during 20 generations of growth. More generally, the LF value can be calculated as follows:

$$\nu_1 = (1 - LF)^{g_1} \text{ and } \nu_2 = (1 - LF)^{g_2}$$

where ν_1 and ν_2 are the frequency of plasmid bearing cells after g_1 and g_2 generations, repectively and LF is the frequency of loss per cell per generation. Hence, it follows that

$$\nu_1/\nu_2 = (1 - LF)^{g_1-g_2}$$

and

$$LF = 1 - (\nu_1/\nu_2)^{(1/(g_1-g_2))}$$

Using this formula, errors because of fluctuations in the number of inoculating cells at time zero are avoided. A more convenient approximation of this formula is

$$LF = \ln(\nu_1/\nu_2)/(g_2-g_1)$$

Incompatibility test.

Plasmids believed to carry an inserted *par* region were screened for by utilizing the observation that two otherwise compatible replicons carrying the *same par* region are incompatible with one another, leading to the loss of either of the two in the absence of selection pressure. The test is performed by transforming the plasmid to be tested to a bacterial strain carrying another plasmid, selecting for both plasmids on double selective plates. After streaking on a double selective plate (a plate containing two different antibiotics), the incompatibility was measured either qualitatively or quantitatively.

For the qualitative incompatibility test, a colony from the double selective plate was streaked on a LA plate to form single colonies. About 10 colonies from this plate were resuspended in 1 ml of 0.9% NaCl and diluted to 10^{-4} and 10^{-5}, respectively. 0.1 ml of the 10^{-4} and 10^{-5} dilutions were spread on LA plates. From these plates, 50 colonies (or 200 colonies if a weak incompatibility was expected) were tested on the appropiate selective plates. If a Lac^+ plasmid was in-

cluded in the test, McConkey lactose indicator plates were used instead of replica plating on selective plates. In the case of Lac^+ plasmids, the screening was thus performed by transforming suspected Par^+ plasmids to a strain already harbouring a Par^+ hybrid plasmid mediating a Lac^+ phenotype. Plasmid incompatibility, and consequently proof of a specific Par^+ phenotype of the incoming plasmid is easily detected by screening for Lac^- colonies on McConkey plates, showing that the resident Par^+ plasmid had been destabilized. An example of this screening procedure is described in Example 4.

Quantitative incompatibility measurements were carried out by measuring the loss frequencies of Lac^+ plasmids after establishing heteroplasmid populations as described above. The LF values were measured as described under "Partitioning test III".

Genetic techniques.

Transformation of bacteria was done according to Cohen et al., *Proc. Natl. Acad. Sci. USA* 62, 1972, pp. 2110-2114, with the modification that when a low transformation frequency was expected, the treatment of the competent cells with DNA on ice was prolonged several hours and after heat shock, the cells were again cooled on ice for 5-30 minutes. This treatment increased the transformation frequency significantly.

Infection with the bacteriophage λ was performed by growing 10 ml cultures overnight in LB medium supplemented with 0.2% of maltose (to induce the *malB* protein which is the λ receptor) with shaking. The cells were washed with 0.9% of NaCl and resuspended in 2 ml 0.01 M $MgSo_4$ and incubated for 1 hour. Dilutions of the λ suspension (10^0, 10^{-1}, 10^{-2}) were prepared and 0.1 ml of the phage dilution was used to infect 0.2 ml of the suspension of starved cells. The 10^0, 10^{-1} and 10^{-2} dilutions of the infection mixtures were spread on selective plates.

To transpose plasmids with Tn5 (Km^R) the source of which was the phage λ b221::Tn5, which had been deleted of the attachment site, so

that it was unable to lysogenize, a suspension of the phage was used to infect cells harbouring the plasmid onto which transposition was wanted. The infection was performed as described above and kanamycin resistant cells were selected. Several thousand Km^R colonies were collected and 0.1 ml of a 10^{-2} dilution of these was used to inoculate 100 ml LB medium. The culture was grown overnight and plasmid DNA prepared from this mixture of cells and used to transform *E.coli* K12 strain CSH50 to kanamycin resistance.

Table 2

Plasmids and Phages

Plasmid/ Phage	Source
pKN184	Nordström et al., *Plasmid 4*, 1980, p. 322.
pSF2124	So et al., *Mol. Gen. Genet. 142*, 1975, pp. 239-49.
pJL99	Light & Molin, *Mol. Gen. Genet. 184*, 1981, pp.56-61.
pGA46	An & Friesen, *J. Bact. 140*, 1979, pp. 400-407.
pKN501	Molin et al., *J. Bact. 138*, 1979, pp. 70-79.
pF1403-11	Constructed by inserting the *AluI-NaeI* fragment from the basic replicon of plasmid F in the *SmaI* site of pMC1403 (Casadaban et al., *J. Bact. 143*, 1980, p. 971), creating a fusion between the *E* gene of plasmid F and the *lacZ* gene of pMC1403.
pHP34	Prentki et al., *Gene 17*, 1982, pp. 189-96.
pMC903	Casadaban et al., *J. Bact. 143*, 1980, p. 971.
pKN1562	Molin et al., *J. Bact. 138*, 1979, pp. 70-79.
pVH1424	Constructed by P. Valentin-Hansen by inserting a *Sau*3A fragment carrying the *deo* promoter from plasmid pVH17 (Valentin-Hansen et al., EMBO J., 1982, 317) in the *Bam*HI site of plasmid pMC1403 (Casadaban et al., *op. cit.*, 971).
pSKS104	Constructed by M. Casadaban by inserting a *PvuII* fragment containing the *lac* promoter and translation initiation region from pM13mp7 (Messing et al.,

	Nucleic Acids Res. *9*, 1981, 309) in the *Sma*I site of pMC1403, followed by homologous recombination between the *lacZ* segments.
pBR322	Bolivar et al., *Gene 2*, 1977, p. 95.
pMB1	Betlach et al., *Fed. Proc. 35*, 1976, pp. 2037-43.
λb221::Tn5	Berg, D.E., "Insertion and excision of the transposable kanamycin resistance determinant Tn5", in *DNA Insertion Elements, Plasmids and Episomes* (ed. A.I. Bukhari et al.).
EDλ4	Dempsey and Willetts, *J. Bact. 126*, 1976, p. 166.

EXAMPLE 1

Insertion of Tn5 (Km^R) in the EcoR1-A fragment from R1

Cells of *E.coli* K-12 strain CSH50 harbouring plasmid pKN184, a plasmid which consists of plasmid pSF2124 and the 19 kb *Eco*R1-A fragment (carrying the *par*A and *par*B regions) from plasmid R1 (Nordström et al., *Plasmid 4*, 1980, p. 322), were infected as described under Materials and Methods with a suspension of bacteriophage λb211::Tn5. After selection for kanamycin resistance on LA plates containing 200 µg/ml of kanamycin, colonies were collected and mixed. 0.1 ml of a 10^{-2} dilution of these was used to inoculate 100 ml LB medium. The culture was grown overnight, and plasmid DNA prepared from the culture was used to transform *E.coli* K-12 strain CSH50 selecting for kanamycin resistance on plates containing 200 µg/ml of kanamycin.

In this way, a plasmid, pOU1, was found which mediates resistance to kanamycin and has an insertion of phage DNA corresponding to about 5 kb (5000 base pairs). The plasmid had a molecular weight/size of 34 kb measured by preparing plasmid DNA and analyzing on agarose gels and the following phenotype: Km^R, Ap^R, $ParA^+$, $ParB^+$.

Plasmid DNA was prepared from pOU1 and the plasmid mapped with restriction enzymes by purifying the plasmid DNA, cleaving it with restriction enzyme(s) and analyzing the resulting fragments by means of agarose gel electrophoresis (cf. Fig. 1). In this way, the λ::Tn5 fragment inserted in the *Eco*R1-A fragment of pKN184 was identified as carrying the gene coding for Km^R, and the location of the fragment was determined as shown in Fig. 1. Plasmid pOU1 was used for further analysis and plasmid constructions.

The strain of *E.coli* CSH50/pOU1 is deposited in the German Collection of Microorganisms (Deutsche Sammlung von Mikroorganismen, Grisebachstrasse 8, D-3400 Göttingen), in the following abbreviated to DSM, under the Accession No. 2712.

EXAMPLE 2

Construction of a plasmid useful for cloning of par+ fragments

Plasmid pJL99 (Light & Molin, *Mol. Gen. Genet. 184*, 1981, pp. 56-61) carries a fusion between the *repA* gene from plasmid R1 and the *lac* operon; the plasmid mediates a Lac+ phenotype. The *PstI-SalI* fragment carrying the *repA* - *lac* fusion was excised from pJL99 and inserted in pGA46 which is a p15 replicon (An & Friesen, *J. Bact. 140*, 1979, pp. 400-407) to construct plasmid, pJL124. A map of the plasmid is shown in Fig. 2. Plasmid pJL124 also mediates a Lac+ phenotype on McConkey lactose indicator plates, but it was found that insertion of DNA fragments with little or no promoter activity in the *PstI* site of pJL124 interferes with the activity of the *repA* promoter in such a way that these hybrids no longer show up as Lac+ on McConkey lactose indicator plates. However, on the more sensitive X-gal indicator plates, transformant colonies are Lac+ indicating that expression of β-galactosidase is reduced, but not entirely suppressed in the hybrids. Therefore, pJL124 can be used to clone *PstI* fragments since hybrid plasmids are easily detected on McConkey lactose indicator plates as Lac- transformants. Moreover, since pJL124 is a p15 replicon and thus unstable, which is easily detected as cells

from which the plasmid has been lost form colourless colonies on lactose indicator plates without selection pressure, *Pst*I fragments able to stabilize pJL124 can be screened for on X-gal plates.

The programme for isolating *Pst*I - *par*⁺ fragments thus consists of the following steps:

1) Ligation of pJL124 restricted with *Pst*I and *Pst*I fragments from another plasmid.
2) Transformation to CSH50 plating on McConkey-lactose plates containing chloroamphenicol.
3) Testing clones that show up as Lac^- on McConkey-lactose plates for stable inheritance of Lac^+ phenotype on X-gal indicator plates (cf. Materials and Methods).

The strain *E. coli* CSH50/pJL124 is deposited in the DSM under the Accession No. 2760.

EXAMPLE 3

Stabilization of unstable plasmids with the parA and parB regions

1. Mini-R1 replicon

Plasmid pOU71 (DSM Accession No. 2471), a runaway replication derivative of plasmid pKN1562 which comprises the basic replicon of plasmid R1, the λP_R promoter and cI_{857} repressor gene from phage EDλ4, the gene coding for β-lactamase from the Tn3 transposon and a unique *Eco*R1 site (a detailed description of the construction of pOU71 is found in Applicant's co-pending application entitled, "Plasmids with Conditional Uncontrolled Replication Behaviour" and filed on the same day as the present application), was restricted with *Eco*R1, and the *Eco*R1-A::Tn5 fragment from pOU1 (cf. Example 1) was inserted followed by ligation and transformation of *E.coli* strain CSH50, selecting of kanamycin resistant cells on LA plates containing 50µg/ml of kanamycin.

The transformants were screened for the runaway replication phenotype of pOU71 by streaking on LA plates containing 50 µg/ml of kanamycin and incubating at 42°C. Cells containing runaway replication plasmids eventually cease to grow under these conditions. In this way, plasmid pOU71-184 was identified, which had a size of 25.5 kb and the following phenotype: ParA+, ParB+, Ap^R, Km^R.

The plasmid was mapped with restriction enzymes as described in Example 1 (cf. Fig. 3). From the restriction map it appears that the *Eco*R1-A::Tn5 fragment has been inserted correctly in the *Eco*R1 site of pOU71.

Cells harbouring the plasmid were grown on LA plates for 100 generations without selection pressure, i.e. without being grown on plates containing an antibiotic; when determined according to the procedure described in Materials and Methods (Partitioning Test II), the cells were not observed to lose the plasmid, whereas pOU71 was lost from cells grown under similar conditions with a frequency of 1% per generation. Thus, it was determined that pOU71-184 is stably inherited with a frequency of loss of about 10^{-6}/cell/generation.

The strain of *E.coli* CSH50/pOU71-184 is deposited in the DSM under the Accession No. 2763.

2. p15 replicon

Plasmid pJL124 (cf. Example 2), which is a Par^- p15 replicon, was cleaved with the restriction enzyme *Pst*I and mixed with plasmid pKN184 (cf. Example 1) which had been partially restricted with *Pst*I followed by ligation. The ligation mixed was transformed to *E.coli* strain CSH50 selecting for chloramphenicol resistance on McConkey lactose indicator plated containing 50 µg/ml of chloramphenicol.

Cells harbouring pJL124 carrying one or more *Pst*I fragments (cf. Example 2) were tested for stable inheritance of the Lac+ phenotype on X-gal plates. One of the stably inherited plasmids was found to contain both the *parA* and the *parB* region. This plasmid, pOU2, has

a size of 24 kb and the following phenotype: Cm^R, Lac+, ParA+, ParB+.

The plasmid was mapped with restriction enzymes as described in Example 1 (cf. Fig. 4). From the restriction map it appears that a *Pst*I fragment has been deleted from the *Eco*R1-A fragment.

Cells harbouring the plasmid were grown on X-gal plates for 100 generations without selection pressure, and it was determined that pOU2 is stably inherited with an LF-value of less than 10^{-6}/cell/-generation in contrast to pJL124 which was lost from the cells with a frequency of 0.5-1%/cell/generation.

The strain *E.coli* CSH50/pOU2 is deposited in the DSM under the Accession No. 2713.

3.pMB1 replicon

Plasmid pF1403-11 (cf. Table 2) is an unstably inherited derivative of pBR322 (a pMB1 replicon) carrying a fusion between a gene from plasmid F and the *lac* operon. The plasmid mediates an Ap^R, Lac+ phenotype. The *Eco*R1-A::Tn5 fragment from plasmid pOU1 (Example 1) was inserted in the unique *Eco*R1 site of plasmid pF1403-11 immediately upstream of the gene fusion followed by ligation and transformation to *E.coli* strain CSH50 selecting for Ap^R on plates containing 50 µg/ml ampicillin, Km^R on plates containing 50 µg/ml kanamycin and Lac+ on McConkey lactose indicator plates.

The resulting plasmid pOU10 was mapped with restriction enzymes as described in Example 1 (cf. Fig. 5), and the insertion of the *Eco*R1-A::Tn5 fragment was verified. The plasmid has a size of 34 kb and the following phenotype: Km^R, Ap^R, Lac+, ParA+, ParB+.

The plasmid was tested for stable inheritance as described in Example 1. Thus, it was demonstrated that pOU10 is stably inherited with an LF-value of less than 10^{-6}/cell/generation, whereas pF1403-11 was lost from the cells with a frequency of 6×10^{-3} per generation.

The strain *E.coli* CSH50/pOU10 is deposited in the DSM under the Accession No. 2714.

EXAMPLE 4

Cloning of PstI fragments from the EcoR1-A fragment which stabilizes pJL124

The *Eco*R1-A fragment was restricted with a number of restriction enzymes, and the resulting physical map is shown in Fig. 1. As appears from this figure, the fragment is composed of many *Pst*I fragments. In order to reduce the size of the fragment mediating the Par$^+$ phenotype, it was attempted to subclone *par* regions possibly carried on one or more of the *Pst*I fragments in the screening vector pJL124 (cf. Example 2). Analysis of a number of clones with the correct phenotype - Lac$^-$ on McConkey lactose plates and stable inheritance in the absence of selection pressure - showed that the *Pst*I-D fragment (cf. Fig. 1) mediated this phenotype. No other single *Pst*I fragment was able to stabilize pJL124. The region responsible for stabilization of pJL124 located within the 1.8 kb *Pst*I fragment was denoted *parB*.

In order to analyse the *Pst*I-D fragment further, the fragment was cloned into the unique *Pst*I site in the *bla* gene of pBR322 resulting in pOU93 (cf. Fig. 7; the plasmid is deposited in the DSM under the Accession No. 2724). Restriction mapping of this plasmid showed that the *Pst*I-D fragment contains three *Rsa*I sites (cf. Fig. 6). *Rsa*I generates blunt ends, and the 900 bp *Rsa*I fragment was therefore inserted in the *Sma*I site of plasmid pHP34 (Prentki et al., *Gene 17*, 1982, pp. 189-96) in the following way. pOU93 was restricted with *Rsa*I and mixed with pHP34 restricted with *Sma*I followed by ligation. The ligation mixture was transformed to *E.coli* strain CSH50 already harbouring plasmid pOU94 (a p15 derivative which is phenotypically Lac$^+$ and ParB$^+$; cf. Fig. 8; the plasmid is deposited in the DSM under the Accession No. 2725), selecting for ampicillin resistance on plates containing 50 μg/ml of ampicillin.

Due to the incompatibility expressed by *parB*+ regions carried on different plasmids, pOU94 is lost when another plasmid which is phenotypically ParB+ is introduced into the *E.coli* cells, thus making it possible to select for correct insertions and transformants by streaking the cells onto McConkey plates and screening for colourless colonies (Lac^-). One such incompatible plasmid pHP34 derivative which was found to contain the 900 bp *Rsa*I fragment was denoted pOU13. The plasmid has a size of 5.3 kb and the following phenotype: Tc^R, Ap^R, ParB+.

The plasmid was mapped with restriction enzymes as described in Example 1 (cf. Fig. 9). The mapping showed that the *Rsa*I fragment had been converted to a 900 bp *Eco*R1 fragment as the *Sma*I site of pHP34 is flanked by two *Eco*R1 sites.

The strain of *E. coli* CSH50/pOU13 is deposited in the DSM under the Accession No. 2716.

The 900 bp *Eco*R1 fragment from pOU13 was inserted in the *Eco*R1 site of pOU101, an Ap^R, Cm^R runaway mini-R1 derivative (a detailed description of the construction of pOU101 is found in Applicant's co-pending application entitled, "Plasmids with Conditional Uncontrolled Replication Behaviour" and filed on the same day as the present application) with a unique *Eco*R1 site in the *cat* gene (the gene coding for Cm^R), to construct plasmid pOU14 which has a size of 8.2 kb and the following phenotype: Cm^S, Ap^R, ParB+.

The plasmid was mapped with restriction enzymes as described in Example 1 (cf. Fig. 10).

The plasmid was tested for stable inheritance as described in Example 3.1. Thus, it was demonstrated that, at 30°C, pOU14 is stably inherited with an LF-value of less than 1 x 10^{-4}/cell/generation, whereas pOU101 is lost from the cells with a frequency of 1% per generation.

The strain of *E. coli* CSH50/pOU14 is deposited in the DSM under the Accession No. 2717.

EXAMPLE 5

Stabilization of mini-R1 plasmids with the parB fragment

Plasmid pOU90, a runaway replication derivative of pKN1562 which comprises the basic replicon of plasmid R1, the λP_R promoter and cI_{857} repressor from phage EDλ4, the gene coding for β-lactamase from the Tn3 transposon and the *Eco*R1-A fragment from pKN184 (a detailed description of the construction of pOU90 is found in Applicant's co-pending application entitled: "Plasmids with Conditional Uncontrolled Replication Behaviour" and filed on the same day as the present application), in which derivative the *Eco*R1-A fragment from pKN184 has been inserted, was restricted with *Sal*I and ligated to produce plasmid pOU61. The plasmid was transformed to *E.coli* strain CSH50 selecting for a Lac^- phenotype on McConkey plates.

pOU61 was mapped with restriction enzymes as described in Example 1 (cf. Fig. 11). From the restriction map it appears that pOU61 carries the 3.6 kb right end of the *Eco*R1-A fragment containing the *parB* region. The plasmid has a size of 10 kb and the following phenotype: $ParB^+$, Ap^R, Lac^-. The plasmid was tested for stable inheritance as described in Example 3.1. Thus, it was demonstrated that pOU61 is stably inherited with an LF-value of less than 1×10^{-4}/ cell/generation.

The strain of *E. coli* CSH50/pOU61 is deposited in the DSM under the Accession No. 2723.

EXAMPLE 6

Stabilization of plasmid pF1403-11 with the parB fragment

Plasmid pOU1 (cf. Example 1) was restricted with *Sal*I and mixed with plasmid pF1403-11 which had also been restricted with *Sal*I followed by ligation and transformation to *E. coli* strain CSH50, selecting for ampicillin resistant colonies on McConkey lactose indicator plates. Some of these Lac^{+} clones were screened for stable inheritance of the Lac^{+} phenotype on McConkey plates as described in Materials and Methods.

The stably inherited plasmids were mapped with restriction enzymes as described in Example 1 (cf. Fig. 12). From the restriction map it appears that they carry the *Sal*I fragment of pOU1 on which the *parB* region is located. The plasmid consisting of pF1403-11 and the *Sal*I fragment from pOU1 was designated pOU12. It had a size of 16 kb and the following phenotype: Par B^{+}, Lac^{+}, Ap^{R}. pOU12 was stably inherited with an LF value of less than 5×10^{-5}/cell/generation.

The strain of *E. coli* CSH50/pOU12 is deposited in the DSM under the Accession No. 2715.

EXAMPLE 7

Stabilization of p15 plasmids with the parA fragment

Plasmid pMC 903 (Casadaban et al., *J. Bact. 143*, 1980, p. 971) was restricted with *Eco*R1, and the *Eco*R1 fragment from plasmid pOU43 (cf. Fig. 13; DSM Accession No. 2720) carrying the *parA* region was inserted followed by ligation and transformation to *E.coli* strain CSH50. The resulting plasmid was denoted pOU45 and had a size of 13.4 kb and the following phenotype: $ParA^{+}$, Lac^{-}, Ap^{R}, Km^{R}.

The plasmid was mapped with restriction enzymes as described in Example 1 (cf. Fig. 14). From the restriction map it appears that the *parA* region has been inserted in the *lac* operon which is thereby inactivated.

When tested for stability as described in Materials and Methods, the plasmid was found to be stably inherited with an LF value of 8 x 10^{-4}/ cell/generation, whereas pMC903 has an LF value of 1%/cell/generation.

The strain of *E.coli* CSH50/pOU45 is deposited in the DSM under the Acc. No. 2721.

EXAMPLE 8

Stabilization of mini-R1 plasmids with the parA region

The *Eco*R1 fragment from pOU43 was inserted in a mini-R1 plasmid, pOU82 (DSM Accession No. 2482), which comprises the basic replicon of plasmid R1, the λP_R promoter and cI repressor gene from phage EDλ4, the gene coding for β-lactamase from the Tn3 transposon, the *deo* promoter and aminoterminal end of the *lacZ* gene from pVH1424 and the remainder of the *lac* operon from pSKS104 (a detailed description of the construction of pOU82 is found in the Applicant's copending application entitled "Plasmids with Conditional Uncontrolled Replication Behaviour" and filed on the same day as the present application). Plasmid pOU82 mediates the Ap^R and Lac^+ phenotypes and has a unique *Eco*R1 site useful for cloning *Eco*R1 fragments. This plasmid is lost with a frequency of 1% per generation in the absence of selection pressure.

A plasmid in which the 2.4 kb *Eco*R1 fragment had been inserted in one orientation was denoted pOU47.The plasmid had a size of 14 kb and the following phenotype: $ParA^+$, Lac^+, Ap^R.

pOU47 was mapped with restriction enzymes as described in Example 1 (cf. Fig. 15).

The strain of *E.coli* CSH50/pOU47 is deposited in the DSM under Accession No. 2722.

The *Eco*R1 fragment carrying the *parA* region was further reduced with 400 bp by means of the exonuclease *Bal*31; the deletion procedure was performed as described in Materials and Methods utilizing the unique *Bam*HI site of pOU47. The plasmid constructed was denoted pOU472. The plasmid had a size of 13 kb and the following phenotype: ParA+, Lac+, Ap^R.

pOU472 was mapped with restriction enzymes as described in Example 1 (cf. Fig. 16). From the restriction map it appears that pOU472 carries the 2.0 kb *Eco*R1 fragment generated by the *Bal*31 deletion.

When tested for stability as described in Materials and Methods, the plasmid was found to be stably inherited which means that the entire *parA* region is contained in the 2.0 kb *Eco*R1 fragment.

The strain of *E.coli* CSH50/pOU472 is deposited in the DSM under the Accession No. 2726.

EXAMPLE 9

Construction of a mini-R1 plasmid carrying the parA region

Plasmid pOU90 (cf. Example 5) was cleaved with *Bam*HI and partially with *Sau*3A to delete part of the *Eco*R1-A fragment, the leftmost 6 kb being retained, followed by ligation. The resulting plasmid, pOU91, was transformed to *E.coli* strain CSH50. pOU91 has a size of 18.75 kb and the following phenotype: *Par+*, Ap^R, Lac+. The plasmid was mapped with restriction enzymes as described in Example 1 (cf. Fig. 17).

Plasmid stability was determined by growing cells containing pOU91 (and, as a control, cells containing pOU82) on McConkey plates without selection pressure at 30°C for 25 generations; the cells transformed with pOU91 generated red colonies (Lac+), while cells transformed with pOU82 generated both red and white colonies indicating that during the selection-free period, pOU82 had been lost from some of the cells.

The strain of *E.coli* CSH/pOU91 is deposited in the DSM under the Accession No. 2483.

EXAMPLE 10

Construction of a parA+, parB+ mini-R1 plasmid

Plasmid pOU82 (cf. Example 8) is restricted with *Eco*R1, and the *Eco*R1 fragment from pOU43 (cf. Example 7 and Fig. 13) carrying the *parA* region is inserted. This *Eco*R1 fragment is deleted of the left-most 500 bp, including one *Eco*R1 site, by means of the exonuclease *Bal*31. In the unique *Eco*R1 site of this plasmid, the *Eco*R1 fragment from pOU13 (cf. Example 4 and Fig. 9) is inserted. The resulting plasmid, which mediates a ParA+, ParB+ phenotype, may then be transformed to *E. coli* strain CSH50 already carrying plasmid pOU94 and screened for substantially as described in Example 4 with the exeption that the pOU82 derivative mediates a weak Lac+ phenotype which means that colonies of cells harbouring this plasmid only will be red in the center and colourless at the edges when grown on Mc-Conkey lactose indicator plates.

EXAMPLE 11

Construction of a parA+, parB+ mini-R1 plasmid

Plasmid pOU61 (cf. Example 5) is restricted with *Eco*R1, and the *Eco*R1 fragment from pOU43 carrying the *parA* region is inserted followed by ligation. The resulting plasmid, which mediates a ParA+, ParB+ phenotype, may then be transformed to *E. coli* strain CSH50 which already carries plasmid pOU2 and screened for in a manner corresponding to the procedure described in Example 4. Colonies of cells harbouring the desired plasmid will appear as colourless colonies (Lac$^-$) on McConkey lactose plates.

(Throughout the present specification and claims, where par^-, Par^-, par^+ or Par^+ is not specifically indicated, the terms *par* and Par denote the expression of a partitioning function).

CLAIMS

1. A plasmid which carries an inserted gene or genes not naturally related to the plasmid as well as an inserted DNA fragment which expresses a partitioning function.

2. A plasmid according to claim 1 in which the partitioning function is exerted by an R1 *par* region.

3. A plasmid according to claim 2, in which the DNA fragment comprises, as its major component, R1 *par* region A, the R1 *par* region B or both R1 *par* region A and R1 *par* region B.

4. A plasmid according to claim 3 in which the inserted DNA fragment comprising R1 *par* region A and R1 *par* region B has a length not exceeding about 6 kb, especially not exceeding about 4 kb, in particular not exceeding 3 kb.

5. A plasmid according to claim 3, in which the inserted DNA fragment comprising the R1 *par* region A has a length not exceeding about 4 kb, especially not exceeding about 2.5 kb, in particular not exceeding about 2 kb.

6. A plasmid according to claim 3, in which the inserted DNA fragment comprising the R1 *par* region B has a length not exceeding about 2 kb, especially not exceeding about 1.5 kb, in particular not exceeding about 1 kb.

7. A plasmid according to any of claims 1-6 which additionally carries a gene mediating antibiotic resistance.

8. A plasmid according to any of claims 1-7 which in the absence of an inserted DNA fragment exerting a partitioning function would be unstably inherited.

9. A plasmid according to claim 8 which is a p15 plasmid or a derivative thereof, or a high copy number broad host range plasmid or a derivative thereof.

10. A plasmid according to claim 8 which is unstably inherited due to its carrying a DNA fragment comprising a gene or genes not naturally related to the plasmid.

11. A plasmid according to claim 10 which is a pMB1 plasmid or a derivative thereof such as a pBR322 plasmid or a derivative thereof.

12. A plasmid according to claim 8 which, at least at one stage during the cultivation of bacteria harbouring the plasmid, has a low copy number.

13. A plasmid according to claim 12 which has a copy number of about 0.5-5 copies per cell.

14. A plasmid according to claim 12 or 13 which is selected among
plasmids of the incompatibility group IncFII, including R1 and derivatives thereof;
F and derivatives thereof;
and low copy number broad host range plasmids and derivatives thereof.

15. A plasmid according to claim 12 or 13 which is a conditional run-away replication plasmid.

16. A plasmid according to claim 15 which, when host microorganisms containing the plasmid are grown under certain conditions, has a copy number not exceeding about 3-5 copies per cell, and which, when the host microorganisms are grown under certain different conditions, has a copy number in the range of at least about 500-1000 copies per cell.

17. A plasmid according to claim 16 which has a plasmid copy number not exceeding about 3-5 copies per cell at one temperature, and a plasmid copy number in the range of at least about 500-1000 copies per cell at a higher temperature.

18. A plasmid according to claim 16 or 17 in which the plasmid copy number not exceeding about 3-5 copies per cell is in the range of about 0.5-1 copy per cell.

19. A plasmid according to claim 1, 16, 17 or 18 in which a regulatable promoter has been inserted upstream of the native replication control gene(s) of the plasmid.

20. A plasmid according to any of claims 16-18 which is an R1-type plasmid.

21. A plasmid which carries an inserted DNA fragment shorter than the *Eco*R1-A fragment of plasmid R1 and containing an R1 *par* region.

22. A plasmid according to claim 21, in which te inserted DNA fragment comprises the R1 *par* region A, the R1 *par* region B or both R1 *par* region A and R1 *par* region B.

23. A plasmid according to claim 22, in which the DNA fragment comprises, as its major component, R1 *par* region A, the R1 *par* region B or both R1 *par* region A and R1 *par* region B.

24. A plasmid according to claim 23 in which the inserted DNA fragment comprising R1 *par* region A and R1 *par* region B has a length not exceeding about 6 kb, especially not exceeding about 4 kb, in particular not exceeding 3 kb.

25. A plasmid according to claim 23, in which the inserted DNA fragment comprising the R1 *par* region A has a length not exceeding about 4 kb, especially not exceeding about 2.5 kb, in particular not exceeding about 2 kb.

26. A plasmid according to claim 23, in which the inserted DNA fragment comprising the R1 *par* region B has a length not exceeding about 2 kb, especially not exceeding about 1.5 kb, in particular not exceeding about 1 kb.

27. A bacterium which harbours a plasmid according to any of the preceding claims.

28. A bacterium according to claim 27 which is a gram-negative bacterium.

29. A bacterium according to claim 28 which is an *Escherichia coli*.

30. A method for producing a gene product of plasmid DNA, in which bacteria harbouring a plasmid according to any of claims 1-20 or 21-26 are cultivated, and the gene product of the plasmid is harvested from the bacterial culture.

31. A method according to claim 30 in which the cultivation is performed for at least 100 generations of the bacteria.

32. A method according to claim 31 wherein the loss of the plasmid is less than 2×10^{-4}/cell/generation.

33. A method according to claim 32 wherein the loss of the plasmid is less than 10^{-5}/cell/generation.

34. A method according to claim 33 wherein the loss of the plasmid is less than 5×10^{-6}/cell/generation.

35. A DNA fragment which comprises, as its major component, an R1 *par* region.

36. A DNA fragment according to claim 35 which comprises, at its major component, the R1 *par* region A, the R1 *par* region B, or both R1 *par* region A and R1 *par* region B.

37. A DNA fragment according to claim 36 which comprises the R1 *par* regions A and B and which has a length not exceeding about 6 Kb, especially not exceeding about 4 kb, in particular not exceeding 3 kb.

38. A DNA fragment according to claim 36 which comprises the R1 *par* region A and which has a length not exceeding about 4 kb, especially not exceeding about 2.5 kb, in particular not exceeding about 2 kb.

39. A DNA fragment according to claim 36 which comprises the R1 *par* region B and which has a length not exceeding about 2 kb, especially not exceeding about 1.5 kb, in particular not exceeding about 1 kb.

AMENDED CLAIMS
[received by the International Bureau on 24 February 1984 (24.02.84); original claims 1 to 39 unchanged and claims 40 to 44 added]

1. A plasmid which carries an inserted gene or genes not naturally related to the plasmid as well as an inserted DNA fragment which expresses a partitioning function.

2. A plasmid according to claim 1 in which the partitioning function is exerted by an R1 *par* region.

3. A plasmid according to claim 2, in which the DNA fragment comprises, as its major component, R1 *par* region A, the R1 *par* region B or both R1 *par* region A and R1 *par* region B.

4. A plasmid according to claim 3 in which the inserted DNA fragment comprising R1 *par* region A and R1 *par* region B has a length not exceeding about 6 kb, especially not exceeding about 4 kb, in particular not exceeding 3 kb.

5. A plasmid according to claim 3, in which the inserted DNA fragment comprising the R1 *par* region A has a length not exceeding about 4 kb, especially not exceeding about 2.5 kb, in particular not exceeding about 2 kb.

6. A plasmid according to claim 3, in which the inserted DNA fragment comprising the R1 *par* region B has a length not exceeding about 2 kb, especially not exceeding about 1.5 kb, in particular not exceeding about 1 kb.

7. A plasmid according to any of claims 1-6 which additionally carries a gene mediating antibiotic resistance.

8. A plasmid according to any of claims 1-7 which in the absence of an inserted DNA fragment exerting a partitioning function would be unstably inherited.

9. A plasmid according to claim 8 which is a p15 plasmid or a derivative thereof, or a high copy number broad host range plasmid or a derivative thereof.

10. A plasmid according to claim 8 which is unstably inherited due to its carrying a DNA fragment comprising a gene or genes not naturally related to the plasmid.

11. A plasmid according to claim 10 which is a pMB1 plasmid or a derivative thereof such as a pBR322 plasmid or a derivative thereof.

12. A plasmid according to claim 8 which, at least at one stage during the cultivation of bacteria harbouring the plasmid, has a low copy number.

13. A plasmid according to claim 12 which has a copy number of about 0.5-5 copies per cell.

14. A plasmid according to claim 12 or 13 which is selected among
plasmids of the incompatibility group IncFII, including R1 and derivatives thereof;
F and derivatives thereof;
and low copy number broad host range plasmids and derivatives thereof.

15. A plasmid according to claim 12 or 13 which is a conditional run-away replication plasmid.

16. A plasmid according to claim 15 which, when host microorganisms containing the plasmid are grown under certain conditions, has a copy number not exceeding about 3-5 copies per cell, and which, when the host microorganisms are grown under certain different conditions, has a copy number in the range of at least about 500-1000 copies per cell.

17. A plasmid according to claim 16 which has a plasmid copy number not exceeding about 3-5 copies per cell at one temperature, and a plasmid copy number in the range of at least about 500-1000 copies per cell at a higher temperature.

18. A plasmid according to claim 16 or 17 in which the plasmid copy number not exceeding about 3-5 copies per cell is in the range of about 0.5-1 copy per cell.

19. A plasmid according to claim 1, 16, 17 or 18 in which a regulatable promoter has been inserted upstream of the native replication control gene(s) of the plasmid.

20. A plasmid according to any of claims 16-18 which is an R1-type plasmid.

21. A plasmid which carries an inserted DNA fragment shorter than the *Eco*R1-A fragment of plasmid R1 and containing an R1 *par* region.

22. A plasmid according to claim 21, in which te inserted DNA fragment comprises the R1 *par* region A, the R1 *par* region B or both R1 *par* region A and R1 *par* region B.

23. A plasmid according to claim 22, in which the DNA fragment comprises, as its major component, R1 *par* region A, the R1 *par* region B or both R1 *par* region A and R1 *par* region B.

24. A plasmid according to claim 23 in which the inserted DNA fragment comprising R1 *par* region A and R1 *par* region B has a length not exceeding about 6 kb, especially not exceeding about 4 kb, in particular not exceeding 3 kb.

25. A plasmid according to claim 23, in which the inserted DNA fragment comprising the R1 *par* region A has a length not exceeding about 4 kb, especially not exceeding about 2.5 kb, in particular not exceeding about 2 kb.

26. A plasmid according to claim 23, in which the inserted DNA fragment comprising the R1 *par* region B has a length not exceeding about 2 kb, especially not exceeding about 1.5 kb, in particular not exceeding about 1 kb.

27. A bacterium which harbours a plasmid according to any of the preceding claims.

28. A bacterium according to claim 27 which is a gram-negative bacterium.

29. A bacterium according to claim 28 which is an *Escherichia coli*.

30. A method for producing a gene product of plasmid DNA, in which bacteria harbouring a plasmid according to any of claims 1-20 or 21-26 are cultivated, and the gene product of the plasmid is harvested from the bacterial culture.

31. A method according to claim 30 in which the cultivation is performed for at least 100 generations of the bacteria.

32. A method according to claim 31 wherein the loss of the plasmid is less than 2×10^{-4}/cell/generation.

33. A method according to claim 32 wherein the loss of the plasmid is less than 10^{-5}/cell/generation.

34. A method according to claim 33 wherein the loss of the plasmid is less than 5×10^{-6}/cell/generation.

35. A DNA fragment which comprises, as its major component, an R1 *par* region.

36. A DNA fragment according to claim 35 which comprises, at its major component, the R1 *par* region A, the R1 *par* region B, or both R1 *par* region A and R1 *par* region B.

37. A DNA fragment according to claim 36 which comprises the R1 *par* regions A and B and which has a length not exceeding about 6 Kb, especially not exceeding about 4 kb, in particular not exceeding 3 kb.

38. A DNA fragment according to claim 36 which comprises the R1 *par* region A and which has a length not exceeding about 4 kb, especially not exceeding about 2.5 kb, in particular not exceeding about 2 kb.

39. A DNA fragment according to claim 36 which comprises the R1 *par* region B and which has a length not exceeding about 2 kb, especially not exceeding about 1.5 kb, in particular not exceeding about 1 kb.

40. A method of screening for the correct insertion of a *par* region in a plasmid in which a plasmid believed to carry an inserted *par* region is transformed to a bacterium already harbouring another unrelated plasmid carrying the same *par* region as well as mediating a phenotype which is recognisable on appropriate media, the transformed bacterium is grown on such media, and the correct insertion of the *par* region is identified as the loss of this phenotype.

41. A method according to claim 40 in which the recognisable phenotype is an antibiotic resistance.

42. A method according to claim 40 in which the recognisable phenotype is Lac^{+}.

43. A method according to claim 40 in which the *par* region on said other plasmid is *parA*.

44. A method according to claim 40 in which the *par* region on said other plasmid is *parB*.

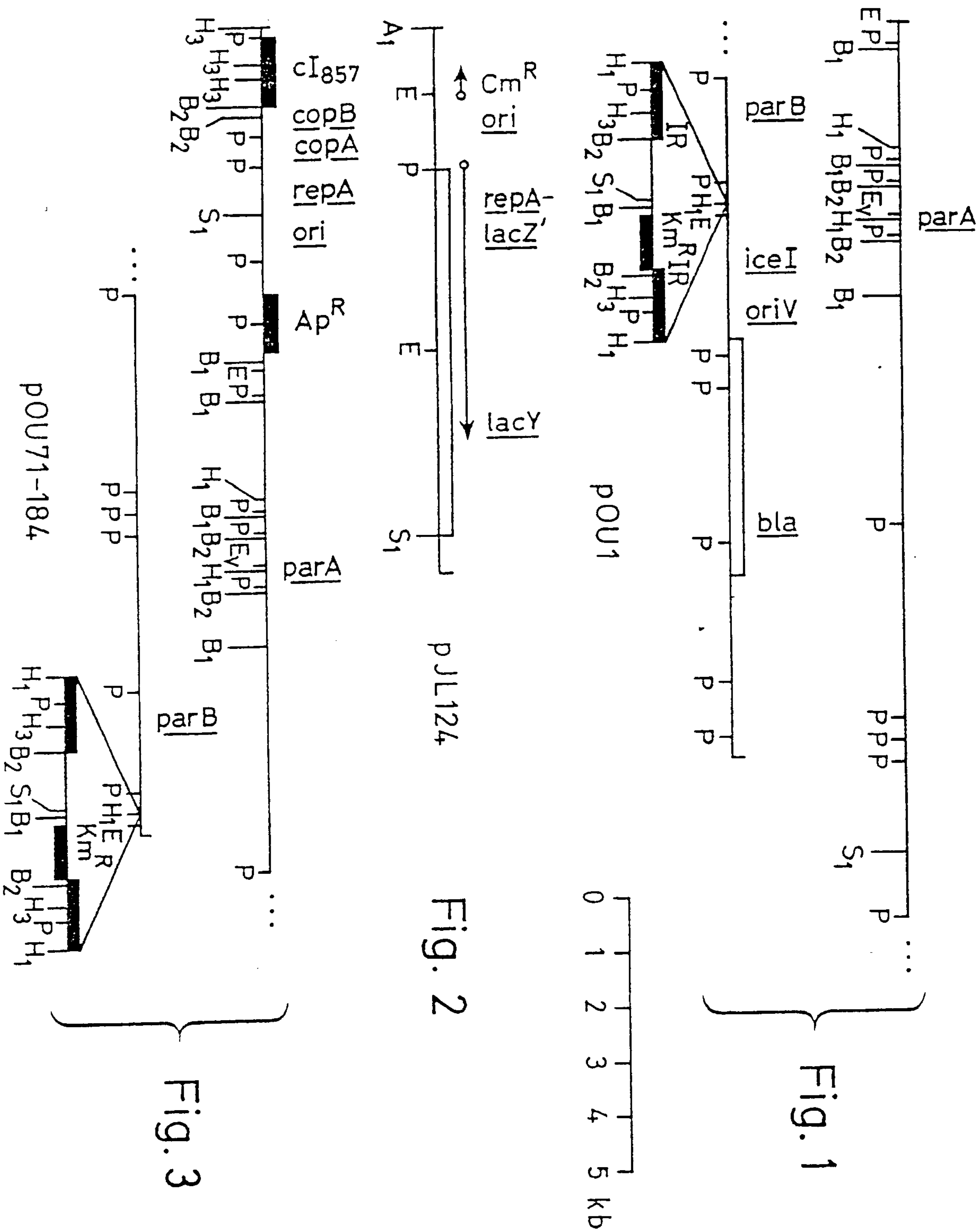


Fig. 1

Fig. 2

Fig. 3

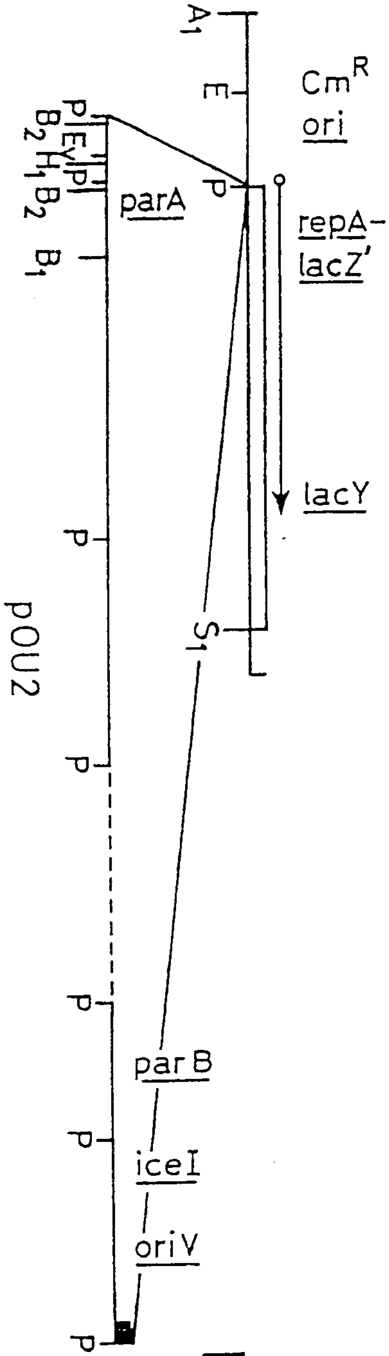


Fig. 4

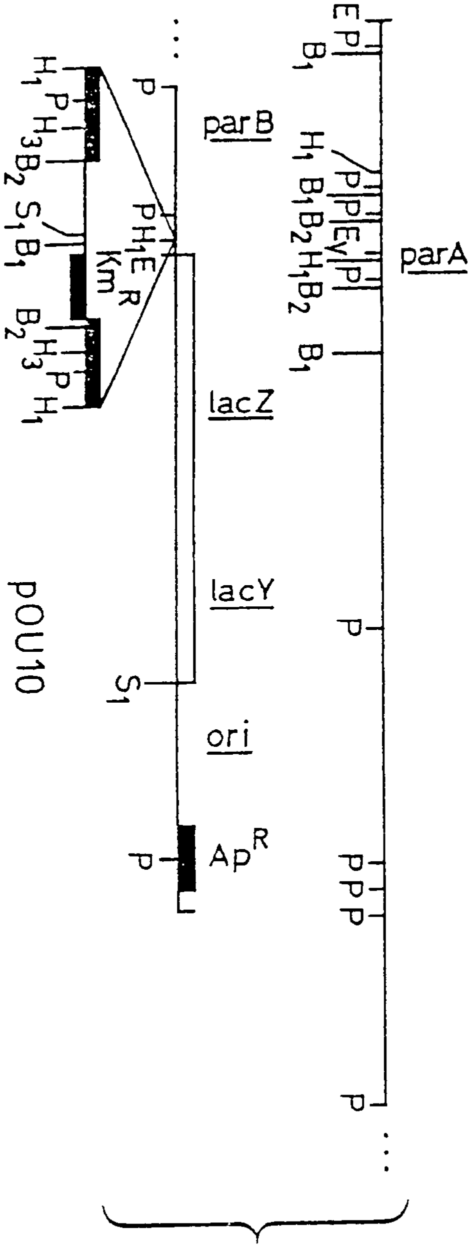


Fig. 5

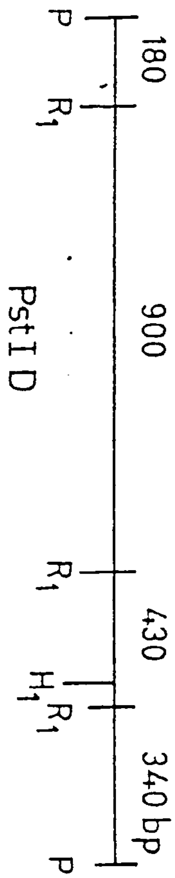


Fig. 6

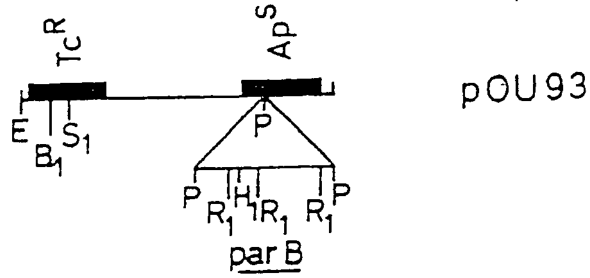


Fig. 7

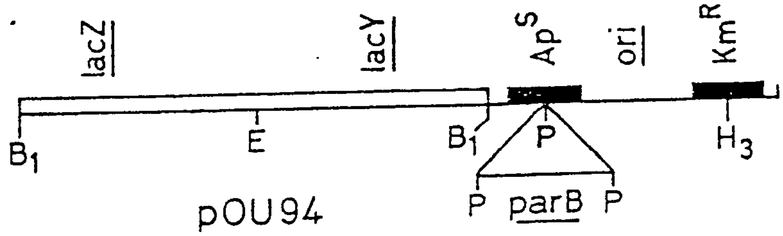


Fig. 8

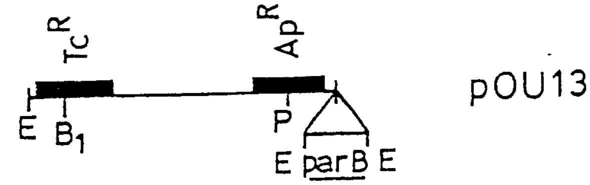


Fig. 9

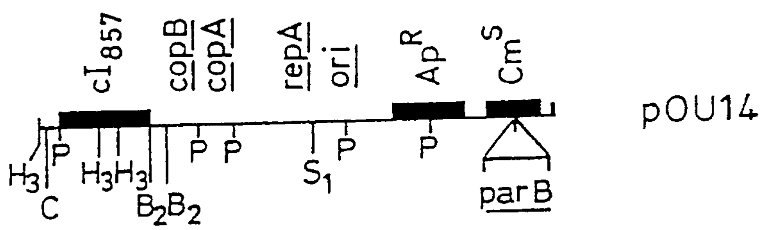


Fig. 10

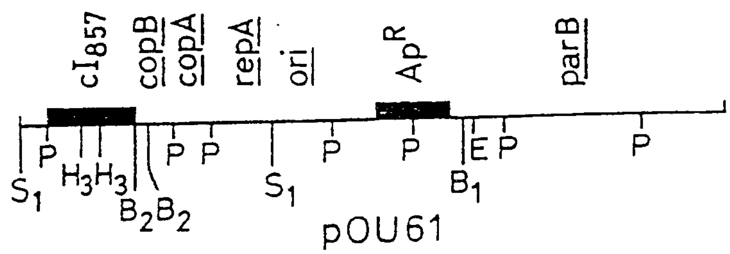


Fig. 11

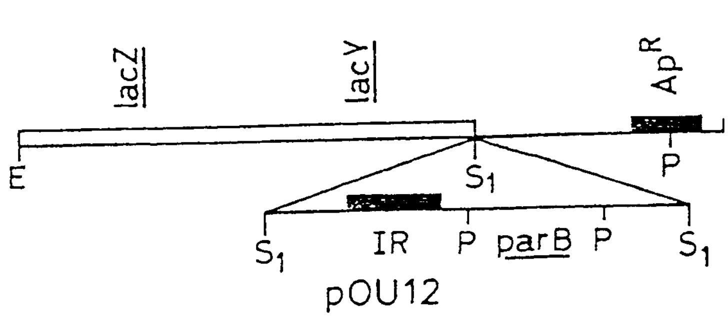


Fig. 12

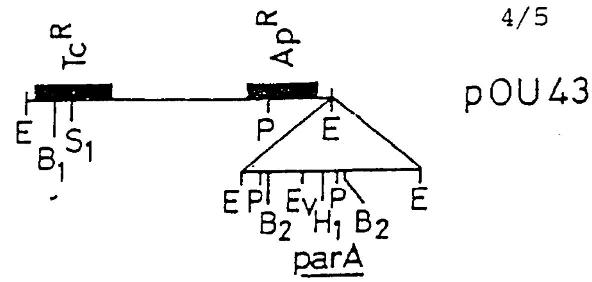


Fig. 13

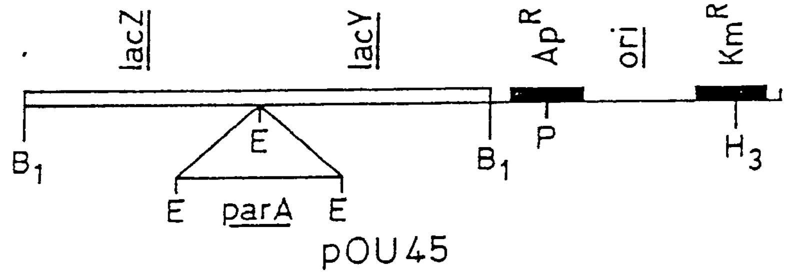


Fig. 14

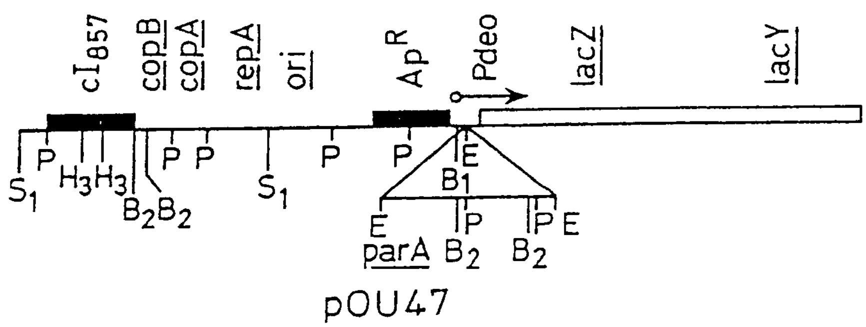


Fig. 15

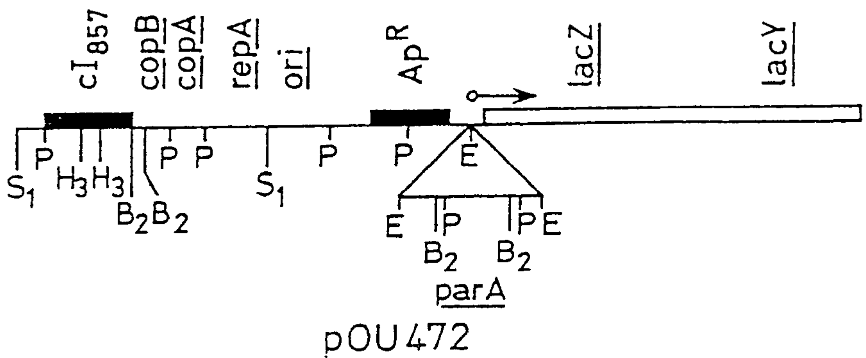


Fig. 16

Fig. 17

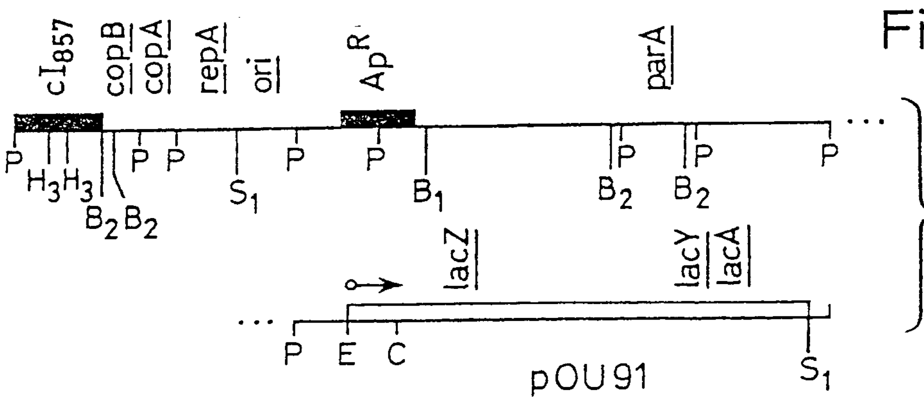
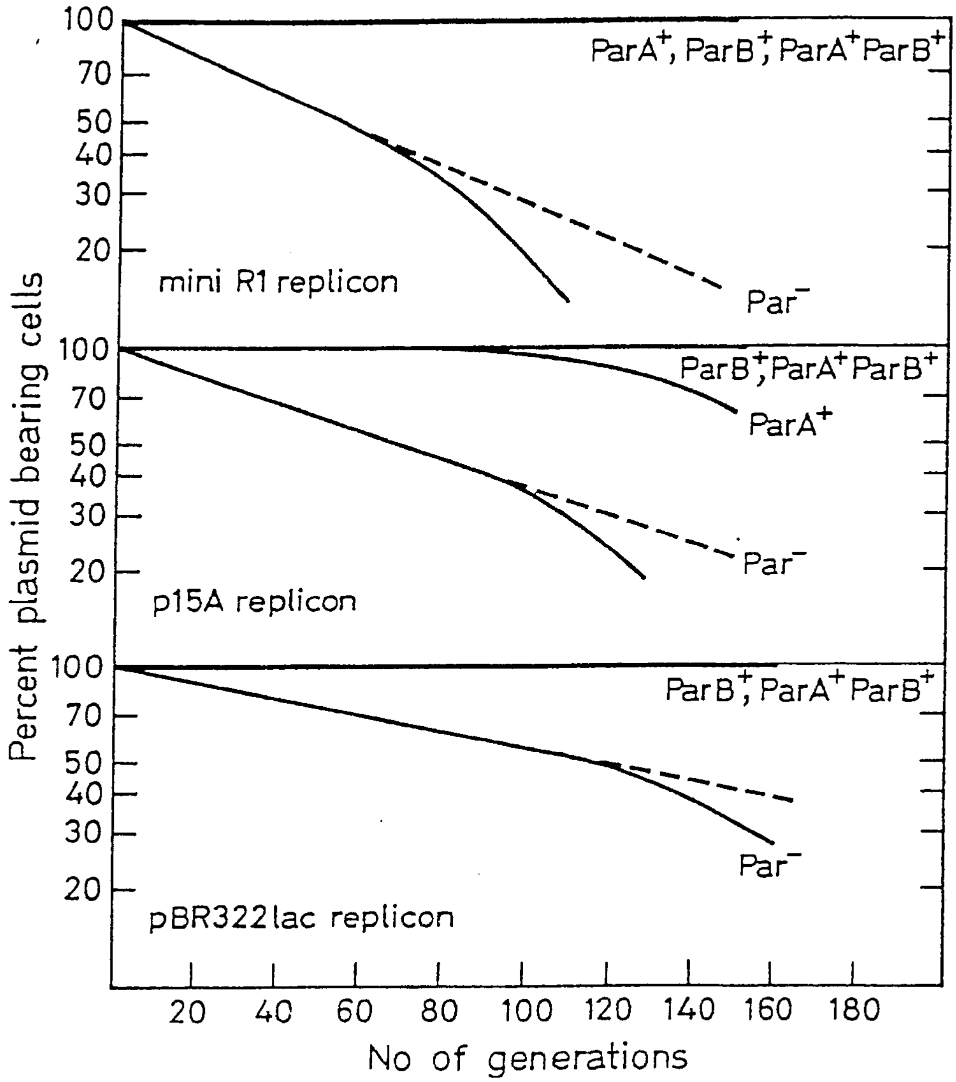


Fig. 18



100
70
50
40
30
20
ParA^{+}, ParB^{+}, ParA^{+}ParB^{+}
mini R1 replicon
Par^{-}
ParB^{+}, ParA^{+}ParB^{+}
ParA^{+}
p15A replicon
Par^{-}
ParB^{+}, ParA^{+}ParB^{+}
pBR322lac replicon
Par^{-}
Percent plasmid bearing cells
20 40 60 80 100 120 140 160 180
No of generations

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK83/00086

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC 3

C 12 N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
IPC 3	C 12 N 15/00
IPC 2	C 12 K 1/02
US Cl	435:172, 317

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	Plasmid, Volume 4(2), published 1980, (K NORDSTRÖM ET AL) see pages 215-227. "Partitioning of plasmid R1 in Escherichia coli. I Kinetics of loss of plasmid derivatives delected of the par region."	1 - 18 20 - 39
X	IVA-PM no 3 published, March 1982, New Hosts and Vectors for r-DNA Technology (P GUSTAFSSON) see pages 75-83 "Genetic system able to increase stability of inheritance of cloning vectors". Symposium February 11, 1982.	1-18 20-39
Y	Cell, Volume 20(2), published 1980, (P A MEACOCK ET AL) see pages 529-542. "Partitioning of bacterial plasmids during cell division: a cis-acting locus that accomplishes stable plasmid inheritance".	1-18 20-39
Y	Plasmid, Volume 7(2), published 1982, (R W SEELKE ET AL) see pages 163-179 "Genetic studies of F plasmid maintenassce genes involved in copy number control, incompatibility, and partitioning" .../...	1-18 20-39

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
1983-12-15	1983 -12- 27
International Searching Authority	**Signature of Authorized Officer**
Swedish Patent Office	Inga-Karin Petersson Inga-Karin Petersson

International Application No. PCT/DK83/00086

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No 18
A	J Bacteriol., Volume 152(1), published 1982 (S AUSTIN ET AL) see pages 63-71 "Genetic and physical map of a P1 miniplasmid".	1-18 20-39

Form PCT ISA 210 (extra sheet) (October 1981)